

## Appendix E

## Identification of a Cytotoxic T Lymphocyte Response to the Apoptosis Inhibitor Protein Survivin in Cancer Patients<sup>1</sup>

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### Abstract

During the last decade, a large number of human tumor-associated antigens have been identified that are recognized by CTLs in a MHC-restricted fashion. The apoptosis inhibitor protein survivin is overexpressed in most human cancers, and inhibition of its function results in increased apoptosis. Therefore, this protein may serve as a target for therapeutic CTL responses. Here, using CTL epitopes deduced from survivin, we describe specific T-cell reactivity against this antigen in peripheral blood from chronic lymphatic leukemia patients and in tumor-infiltrated lymph nodes from melanoma patients by ELISPOT analysis. CTL responses against two survivin-deduced peptide epitopes were detected in three of six melanoma patients and three of four chronic lymphatic leukemia patients. No T-cell reactivity was detected in peripheral blood lymphocytes from six healthy controls. Thus, survivin may serve as an important and widely applicable target for anticancer immunotherapeutic strategies.

### Introduction

It is well established that peptide epitopes derived from TAAs<sup>3</sup> can be recognized as antigens by CTLs in the context of MHC molecules (1). However, although it is generally accepted that most if not all tumors are antigenic, only a few are indeed immunogenic in the sense that tumor progression is readily controlled by the immune system. To overcome this limitation, several immunotherapeutic trials have been initiated, e.g., vaccinations with TAA-derived peptides. For melanoma, the tumor for which the largest number of CTL-defined TAAs have been characterized, powerful CTL responses against antigens have been induced by vaccination, and some patients experienced a complete remission of their disease (2, 3). However, most of the peptide epitopes used in these vaccination trials are melanocyte specific, and these peptides cannot be applied for tumors of nonmelanocyte origin. Furthermore, expression of these TAAs is heterogeneous among tumors from different patients and can vary even among metastases obtained from one patient. However, during the last couple of years, a number of tumor-specific peptide antigens, which are expressed in a number of different cancers, have been identified, i.e., HER-2 (4), Muc-1 (5), and telomerase (6). The use of peptides derived from such proteins could be important in future immunotherapeutic trials.

Apoptosis is a genetic program of cellular suicide, and inhibition of apoptosis has been suggested to be an important mechanism involved

in cancer formation by extending the life span of cells favoring the accumulation of transforming mutations (7). survivin is a recently identified member of the family of inhibitor of apoptosis proteins. In a global gene expression analysis of ~4 million transcripts, survivin was identified as one of the top genes invariably up-regulated in many types of cancer but not in normal tissue (8). Solid malignancies overexpressing survivin include lung, colon, breast, pancreas, and prostate cancer as well as hematopoietic malignancies (9). Furthermore, a series of melanoma and nonmelanoma skin cancers have also been reported to be invariably survivin positive (10, 11). The overexpression of survivin in most human cancers suggests a general role of apoptosis inhibition in tumor progression. This notion is substantiated by the observation that in the case of colorectal and bladder cancer, as well as neuroblastoma, expression of survivin was associated with an unfavorable prognosis. In contrast, survivin is undetectable in normal adult tissues. These characteristics qualify survivin as a suitable TAA for both diagnostic and therapeutic purposes. Thus, we scanned the survivin protein for the presence of HLA-A\*0201 (HLA-A2) binding motifs and, after successful identification, used the peptides to test for specific T-cell reactivity in leukemia and melanoma patients by ELISPOT assay. Indeed, in both patient cohorts, CTL responses against two survivin-derived peptide epitopes were detected, whereas no T-cell reactivity could be detected in the healthy controls. Our data suggest that survivin represents a widely expressed tumor antigen recognized by autologous T cells.

### Materials and Methods

**Patients and Normal Controls.** Peripheral vein blood samples from four patients diagnosed with CLL (designated CLL1–4) and blood samples from six normal individuals were collected into heparinized tubes. PBLs were isolated using Lymphoprep separation and frozen in FCS with 10% DMSO. Furthermore, T lymphocytes from tumor-infiltrated lymph nodes were obtained from six melanoma patients (designated Mel1–6). Freshly resected lymph nodes were minced into small fragments, crushed to release cells into culture, and cryopreserved. PBLs were available from four of the melanoma patients. All individuals included were HLA-A2 positive, as determined by fluorescence-activated cell sorter analysis using the HLA-A2-specific antibody BB7.2. The antibody was purified from hybridoma supernatant. Patient samples were received from the State University Hospital in Herlev. Informed consent was obtained from the patient prior to any of these measures.

**Peptides.** All peptides were purchased from Research Genetics (Huntsville, AL) and provided at >90% purity, as verified by HPLC and MS analysis. All peptides used are listed in Table 1.

**Assembly Assay for Peptide Binding to Class I MHC Molecules.** Assembly assays for binding of the synthetic peptides to class I MHC molecules metabolically labeled with [<sup>35</sup>S]methionine were carried out as described (12, 13). The assembly assay is based on stabilization of the class I molecule after loading of peptide to the peptide transporter-deficient cell line T2. Subsequently, correctly folded stable MHC heavy chains are immunoprecipitated using conformation-dependent antibodies. After IEF electrophoresis, gels were exposed to PhosphorImager screens, and peptide binding was quantitated using the Imagequant PhosphorImager program (Molecular Dynamics, Sunnyvale, CA).

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<sup>3</sup> The abbreviations used are: TAA, tumor-associated antigen; CLL, chronic lymphatic leukemia; PBL, peripheral blood lymphocyte.

Table 1 Peptides examined in this study

Name	Protein <sup>a</sup>	Sequence	C <sub>50</sub> (μM) <sup>b</sup>
C1	HIV-1 pol <sub>476-484</sub>	ILKEPVHGV	0.7
Sur1	survivin <sub>96-104</sub>	LTLGEFLKL	>100
Sur2	survivin <sub>133-141</sub>	RAIEQLAAM	Notbinding
Sur3	survivin <sub>130-138</sub>	KVRRRAIEQL	>100
Sur4	survivin <sub>20-28</sub>	STFKNWPFL	Notbinding
Sur5	survivin <sub>88-96</sub>	SVKKQFEEL	Notbinding
Sur6	survivin <sub>101-109</sub>	FLKLDRELA	30
Sur7	survivin <sub>127-135</sub>	TAKKVRRAI	Notbinding
Sur8	survivin <sub>5-14</sub>	TLPPAWQPFL	30
Sur9	survivin <sub>95-104</sub>	ELTLGEFLKL	10
Sur10	survivin <sub>126-135</sub>	ETAKKVRRAI	Notbinding
Sur1L2		LLLGEFLKL	1
Sur1M2		LMLGEFLKL	1

<sup>a</sup> The value range listed in subscript indicates the position of the peptide in the sequence.

<sup>b</sup> The C<sub>50</sub> value is the concentration of the peptide required for half-maximal binding to HLA-A2.

**Antigen Stimulation of PBLs.** To extend the sensitivity of the ELISPOT assay, PBLs were stimulated once *in vitro* prior to analysis (14, 15). Fresh and previously frozen PBLs gave similar results in the ELISPOT assay. At day 0, PBLs or crushed lymph nodes were thawed and plated in 2 ml/well at a concentration of  $2 \times 10^6$  cells in 24-well plates (Nunc, Roskilde, Denmark) in AIM V medium (Life Technologies, Inc., Roskilde, Denmark), 5% heat-inactivated human serum, and 2 mM of L-glutamine in the presence of 10 μM of peptide. In each experiment, a well without peptide was also included. Two days later, 300 IU/ml recombinant interleukin 2 (Chiron, Ratingen, Germany) were added to the cultures. The cultured cells were tested for reactivity in the ELISPOT on day 12.

**ELISPOT Assay.** The ELISPOT assay used to quantify peptide epitope-specific, IFN-γ-releasing effector cells was performed as described previously (16). Briefly, nitrocellulose-bottomed 96-well plates (MultiScreen MAIP N45; Millipore, Hedehehusene, Denmark) were coated with anti-IFN-γ antibody (1-D1K; Mabtech, Nacka, Sweden). The wells were washed and blocked by AIM V medium, and cells were added in duplicates at different cell concentrations. Peptides were then added to each well, and the plates were incubated overnight. The following day, media were discarded, and the wells were washed prior to addition of biotinylated secondary antibody (7-B6-1-Biotin; Mabtech). The plates were incubated for 2 h and washed, and avidin-enzyme conjugate (AP-Avidin; Calbiochem, Life Technologies, Inc.) was added to each well. Plates were incubated at room temperature for 1 h, and the enzyme substrate nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Life Technologies, Inc.) was added to each well and incubated at room temperature for 5–10 min. The reaction was terminated by washing with tap water upon the emergence of dark purple spots. The spots were counted using the Alpha-Imager System (Alpha Innotech, San Leandro, CA), and the peptide-specific CTL frequency could be calculated from the numbers of spot-forming cells. The assays were all performed in duplicates for each peptide antigen.

## Results

**Binding of Survivin-derived Peptides to HLA-A2.** The amino acid sequence of the survivin protein was screened for the most probable HLA-A2 nona- and deca-mer peptide epitopes, using the main HLA-A2-specific anchor residues (17). Ten survivin deduced peptides were synthesized and examined for binding to HLA-A2. None of the peptides examined bound with similar high affinity as a known positive control epitope from HIV-1 pol<sub>476-484</sub> (ILKEPVHGV; Table 1). The peptide concentration required for half-maximal recovery of class I MHC (C<sub>50</sub>) was 0.7 μM for the positive control. The peptide Sur9 (ELTLGEFLKL) bound in comparison with intermediate affinity (C<sub>50</sub>, 10 μM). The peptides Sur6 (FLKLDRELA) and Sur8 (TLPPAWQPFL) bound weakly to HLA-A2 (C<sub>50</sub>, 30 μM), whereas Sur1 (LTLGEFLKL) and Sur3 (KVRRRAIEQL) bound even more weakly (C<sub>50</sub>, >100 μM). Five of the peptides examined (Sur2, Sur4, Sur5, Sur7, and Sur10) did not bind to HLA-A2. Because Sur1 is a weak HLA-A2 binder, we synthesized two analogue peptides, Sur1L2 and Sur1M2, in which a better anchor residue (leucine

or methionine) replaced the natural threonine at position 2. Both peptides bind with almost similar high affinity to HLA-A2 as the positive control (C<sub>50</sub>, 1 μM).

**CTL Response against Survivin in CLL Patients.** PBLs from four HLA-A2-positive CLL patients were stimulated once *in vitro* before examination in the ELISPOT. This procedure was chosen to extend the sensitivity of the ELISPOT. Because many described CTL epitopes are in fact low-affinity peptides, we included all 10 survivin deduced peptides in the first line of experiments. Responses were detected against Sur1 and Sur9, and only data from these peptides are given in the figures. Fig. 1 shows CTL reactivity against Sur1 and Sur9 as determined in patient CLL1; each spot represents a peptide-reactive, IFN-γ-producing cell. The average number of spots/peptide was calculated using a CCD scanning device and a computer system. Fifty-two Sur9 peptide-specific spots (after subtraction of spots without added peptide) per  $6 \times 10^5$  were detected in the CLL1 patient (Fig. 1B). No response was detected against the weak HLA-A2 binding peptide Sur1; however, the patient responded strongly against the strong HLA-A2 binding peptide analogue Sur1M2 (35 peptide-specific spots/10<sup>4</sup> cells; Fig. 2). No response was detected against the other strong HLA-A2 binding peptide analogue Sur1L2 in this patient (Fig. 2). Patient CLL2 responded strongly against Sur9 (128 peptide-specific spots/10<sup>5</sup> cells) and weakly against Sur1 (22 peptide-specific spots/10<sup>5</sup> cells; Fig. 3). The response against the Sur1L2 analogue was only slightly increased compared with the natural epitope, whereas the patient responded similarly strongly to the Sur1M2 peptide as to the dcamer peptide Sur9. In patient CLL3, we observed only a weak

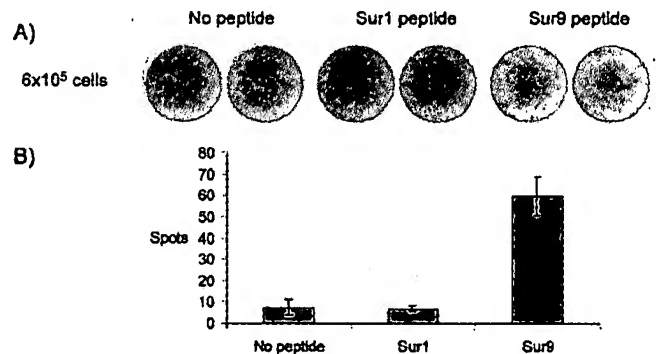


Fig. 1. T-cell response as measured in an ELISPOT in patient CLL1 against no peptide, Sur1 (LTLGEFLKL) peptide, and Sur9 (ELTLGEFLKL) peptide. PBLs were stimulated once with peptide before being plated at  $6 \times 10^5$  cells/well in duplicates (A). The average number of spots/peptide was calculated using a CCD scanning device and a computer system; bars, SD (B).

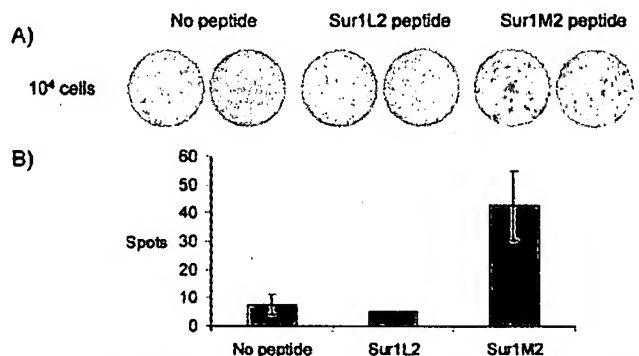


Fig. 2. T-cell response as measured in an ELISPOT in patient CLL1 against no peptide, the peptide analogue Sur1L2 (LLLGEFLKL), and the peptide analogue Sur1M2 (LMLGEFLKL). PBLs were stimulated once with peptide before being plated at  $10^4$  cells/well in duplicates (A). The average number of spots/peptide was calculated using a CCD scanning device and a computer system; bars, SD (B).

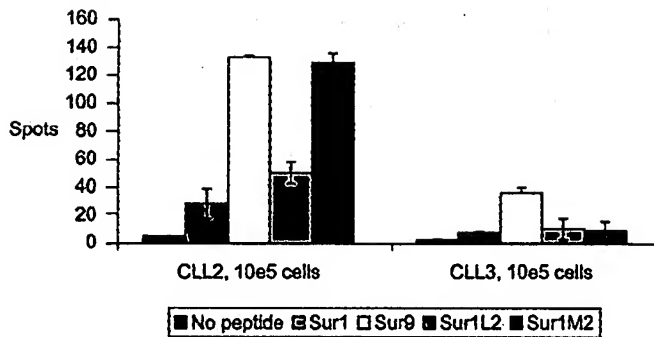


Fig. 3. Responses as measured in an ELISPOT in patients CLL2 and CLL3 against no peptide, the Sur1 (LTLGEFLKL) peptide, the Sur9 (ELTLGEFLKL) peptide, the analogue peptide Sur1L2 (LLLGEFLKL), and the analogue peptide Sur1M2 (LMLGEFLKL). Each experiment was performed with  $10^5$  cells/well in duplicates, and the average number of spots was calculated. Bars, SD.

response against Sur9 (Fig. 3). No response against Sur1 or the modified Sur1 peptides was observed in the patient. No survivin responses were detected in the last patient CLL4 (data not shown). PBLs from six healthy HLA-A2-positive controls were analyzed to investigate whether a response against survivin could be detected in healthy individuals. No response was observed in any of the controls against any of the survivin deduced peptides.

**CTL Response against Survivin in Melanoma Patients.** T lymphocytes isolated from tumor-infiltrated lymph nodes from HLA-A2-positive melanoma patients were examined. The freshly resected lymph node was minced into small fragments and crushed to release cells into culture. Cells were stimulated once with peptide *in vitro* before examination in the ELISPOT. survivin-specific T cells were detected in three of the six patients analyzed. A strong Sur9 response was detected in patients Mel2 and Mel3. A weaker response against the Sur1 peptide was also detected in these patients (Fig. 4). Interestingly, in Mel1 the response against the weak binding peptide Sur1 was stronger than the response against the stronger HLA-A2 binder Sur9 (Fig. 4). No response was detected in the tumor-infiltrated lymph nodes from the last three melanoma patients (Mel4–6). Because of the limited amount of material, it was not possible to examine the response against Sur1L2 or Sur1M2 in the patients. We examined PBLs from two of the survivin-reacting patients, Mel1 and Mel2, and from two of the nonreacting patients, Mel4 and Mel5. No response could be detected against either Sur9 or Sur1 in PBLs from any of these patients (data not shown).

## Discussion

In the present study, we describe that CTLs in both CLL and melanoma patients react against two peptide antigens derived from the apoptosis inhibitor protein survivin. The presence of spontaneous CTL responses against the HLA-A2-restricted peptide antigens derived from survivin in patients suffering from two completely unrelated tumor types, *i.e.*, melanoma and CLL, indicates that these CTL-defined epitopes might be of substantial immunotherapeutic value. Importantly, no CTL response against any survivin-derived, HLA-A2-restricted epitopes was detected in six healthy HLA-A2-positive individuals. Several tumor-specific antigens have been identified, *i.e.*, HER-2 (4), Muc-1 (5), telomerase (6), and viral antigens such as human papillomavirus type 16 (18), and EBV (19); thus, survivin is one additional potentially attractive target for vaccination trials. However, survivin may be of particular interest, because, similar to the catalytic subunit of telomerase, is expressed in most of the common human malignancies. Furthermore, down-regulation or loss

of survivin would severely inflict the growth potential of the tumor cell (9, 20).

Although none of the natural peptides examined bound to HLA-A2 with high affinity, the deca-mer Sur9 (survivin<sub>95–104</sub>, ELTLGEFLKL) bound with intermediate affinity, and the nonamer peptide Sur1 (survivin<sub>96–104</sub>, LTLGEFLKL) bound with low affinity to HLA-A2; we observed spontaneous CTL responses against these two peptides. As could be predicted from their different binding affinities to HLA-A2, the strongest responses were detected against the Sur9 peptide in most of the patients. Because the sequences of the two peptides are very similar, it is likely that T cells cross-react with the two peptides and that the stronger Sur9 response is caused by its higher affinity toward HLA-A2. However, in Mel1, the Sur1 response was stronger than that to Sur9, indicating that a proportion of the T cells, at least in this patient, discriminates between the two peptides. To increase the affinity of the weak HLA-A2 binding peptide Sur1, we modified the peptide by replacing the natural threonine at position 2 with either a leucine or a methionine. This strategy has been used previously to increase binding of peptides to HLA molecules (21–23). Indeed, in two of the CLL patients, the response against the methionine-modified peptide was stronger when compared with that against the native Sur1 peptide. Especially, the very strong response in CLL1 against Sur1M2 is interesting, because no response against the native peptide could be detected in the patient. The use of modified peptides with improved affinities has been demonstrated to be more suitable for the induction of a clinically meaningful CTL response (24).

Many different cancer vaccine approaches have been pursued in a clinical setting during the last couple of years. Recently, treatment with a tumor cell-dendritic cell hybrid vaccine was demonstrated to induce tumor regression in patients with kidney carcinoma (25). It will be of great interest to examine whether survivin-derived peptide epitopes represent one of the targets for the immunological responses induced by this approach. If effective T-cell responses against survivin can indeed be raised in patients, its use in a clinical setting will depend on the type of side effects that may follow immunization. When peptides derived from melanocyte differentiation antigens were

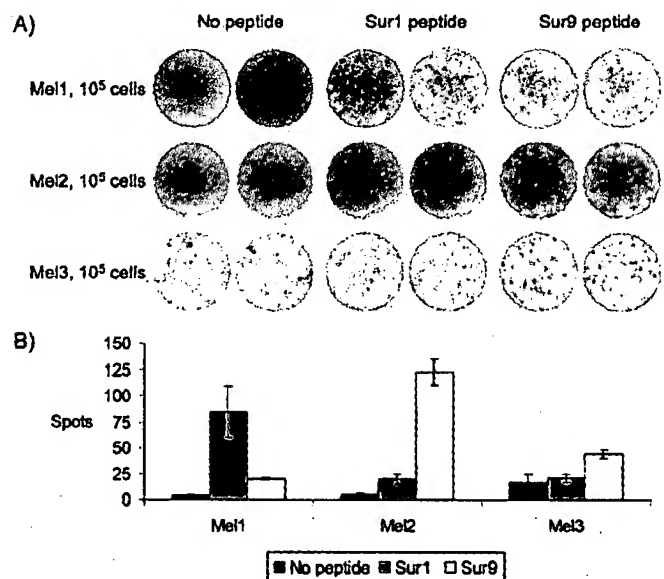


Fig. 4. T-cells were isolated from tumor-infiltrated lymph nodes from patient Mel1 (A, top row), Mel2 (A, middle row), and Mel3 (A, bottom row), stimulated once *in vitro*, and analyzed in an ELISPOT assay against the peptides Sur1 (LTLGEFLKL) and Sur9 (ELTLGEFLKL). Each experiment was performed in duplicates with  $10^5$  cells/well. In each experiment, two wells without addition of peptide was also included (A). The average number of spots/peptide was calculated for each patient; bars, SD (B).

first used to treat patients with stage IV melanoma, it was envisioned that this may lead to pronounced destruction of melanocytes, which in turn might manifest clinically, *i.e.*, vitiligo or retinitis. However, clinical experience demonstrated that the incidence of vitiligo in patients receiving vaccinations is not significantly higher than the incidence of melanoma-associated vitiligo in patients receiving other forms of therapy (26). For survivin, the odds that no major adverse effects in a sense of autoimmunity will be induced are even better because overexpression of survivin is largely restricted to neoplastic cells. Additionally, detectable immune reactions against survivin seem only to be present in tumor patients. The latter notion is not only substantiated by our data but also by a recent report of Rohayem *et al.* (27), describing antibody responses to survivin in up to 20% of tumor patients but not in healthy individuals. Furthermore, neither of the patients included in our study showed any signs of autoimmunity, despite the fact that they hosted a T-cell response against survivin.

In summary, we demonstrate the existence of T-cell responses against two survivin deduced epitopes in cancer patients. Because survivin is abundantly expressed in a variety of other human tumors including lung, colon, breast, prostate, pancreatic, and gastric carcinoma, it is likely that survivin-specific anticancer CTL responses can be detected or introduced in these patients. However, at this time we do not know whether survivin peptides are actually presented by the tumor cells *in vivo*, because the formal proof for this notion is still lacking. Nevertheless, our study gives the first indication toward survivin being a cancer antigen expressed by many different unrelated tumors. The attractiveness of survivin for vaccination purposes is further improved by the fact that down-regulation or loss of its expression as some form of immune escape would hamper the progression of the tumor, particularly if subjected to anticancer chemotherapy.

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